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An examination of scent-marking, individual odors, and individual discrimination in the raccoon (*Procyon lotor*).

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B.S., Animal Science and Animal Ecology, Iowa State University, 2006

A Thesis Submitted to The Graduate School at the University of Missouri - St. Louis in partial fulfillment of the requirements for the degree Master of Science in Biology with an emphasis in Animal Behavior

December 2009

Advisory Committee
Zuleyma Tang-Martínez, Ph.D
Chairperson

George Taylor, Ph.D.

Stan Braude, Ph.D.

General Abstract

This study had three objectives: 1) to characterize the patterns of scent-marking in captive raccoons; 2) to determine if wild-trapped raccoons can discriminate individual differences in the odors of urine, feces or anal sac secretions; 3) to analyze the variation in chemical composition of urine samples among individuals. I observed five raccoons living in a semi-natural enclosure at the Henry Doorly Zoo in Omaha, Nebraska and recorded all behaviors related to scent-marking. For the studies on individual differences and discrimination through odors, I used the familiarization-discrimination technique with raccoons housed in conditions that approximated their natural environment. In the chemical studies, I used gas chromatography to compare qualitative and quantitative differences among individual urine samples. In the zoo study, anogenital rubs were the main form of scent-marking used by raccoons and variation in scent-marking behavior was based on gender but not activity level or dominance. In the odor tests with wild raccoons, the subjects were able to discriminate individual differences between conspecifics in the odors of urine but not feces or anal sac secretions. Chemical analyses showed raccoon urine contains sufficient variability among individuals for individual discrimination. Thus, these data strongly support the hypothesis that raccoons use urine for individual recognition particularly among members of social groups.

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Acknowledgments

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Chapter 1: Scent-marking and Related Behaviors in a Captive Group of Raccoons

Introduction

Communication is considered a prerequisite for the evolution of animal societies (Wilson 1975). Among the various modes of communication, olfactory, or chemical, communication may be the most ubiquitous, being found in a very broad range of taxa, including protists (Newell 1981), invertebrates (Shorey 1976; Wilson 1970), and vertebrates (Shorey 1976; Doty 1976).

Chemical communication has been shown to play a critical role in the social behavior of many mammals (Brown and MacDonald 1985). Studies on olfactory communication in mammalian carnivores have shown that scent-marking has diverse social functions; scent-marks can be indicators of individual identity and carry information about sexual and social status as well as physiological state (Rasa 1973; Gorman 1976; MacDonald 1985). In a captive behavioral study, Holmes (1990) found that chemo-communication in Virginia opossums, *Didelphis virginiana*, may play an important role in coordinating social interactions and that scent-marking in the form of anogenital rubbing, licking and face rubbing may be related to agonistic behavior, fitness, dominance, and priority access to mates. The frequency of scent-marking was also positively correlated with activity level, which may coincide with the increase in activity many animals show during the breeding season when some social odors are of primary importance. In wolves, *Canis lupus*, Asa *et al.* (1985) suggested that conspicuous urine marking may signal dominance in a pack, because only the alpha male and female scent-marked with urine and used raised-leg urination in their study. Other researchers have

found that the function of scent-marking might differ by gender. In the honey badger, *Mellivora capensis*, a solitary carnivore, Begg *et al*. (2003) found that males predominantly placed their scent-marks in communal latrines, whereas females deposited small amounts of urine across different males' territories. North American river otters, *Lontra canadensis,* also use latrines and their feces have been found to contain information on species and sexual identity and, possibly, on dominance status (Karnes and Tumlison 1984; Rostain *et al*. 2004). In the few carnivore species that have been studied, chemical communication appears to be critically important in their social behavior and organization. Consequently, social interactions in a carnivore species cannot be fully understood until the role of chemical communication and most basically, scent-marking patterns and their role in sharing social odors, are explored. Though some species have been investigated, overall the patterns and functions of scent-marking in carnivores are not well understood and knowledge of social odors among the Procyonidae is even more fragmentary than among other carnivore families (MacDonald 1985).

The most well-known member of the family Procyonidae, the raccoon, *Procyon lotor*, has been widely described as a solitary carnivore. One study in North Dakota used radio-tracking and found that adults had large exclusive home ranges (Fritzell 1978). However, the majority of researchers conducting radio-tracking studies, in a range of geographic areas across North America, have found that males and females have overlapping home ranges (Stuewer 1943; Gehrt and Fritzell 1997; Walker and Sunquist 1997; Kamler and Gipson 2003). They have further found that at high and low densities males form coalitions, defend a territory communally, and share resources, dens and

possibly access to females (Gehrt and Fritzell 1998; Pitt *et al.* 2008). This is an unusual form of sociality for a male carnivore and it does not fit with the two common hypotheses to explain home range distribution: resources and access to mates. The large overlapping home ranges of males, sometimes as much as four times larger than the females' home ranges, have been found to be larger than predicted based solely on energetic requirements and resource use (Pitt *et al*. 2008; Gehrt and Fritzell 1997). They also are stable throughout seasons and, thus, not mating-dependent (Gehrt and Fritzell 1997). In addition, coalition formation between males is not directly dependent on the aggregation of females (Pitt *et al*. 2008), nor is it regulated by kin selection as groups are not composed solely of close relatives (Gehrt *et al*. 2008). Therefore, it is not clear what all the factors regulating the home range size of male raccoons are, but it is possible that social interactions, mediated by chemical communication, may plan an important role in socio-spatial behavior in male raccoons.

As stated above, almost nothing is known about chemical communication in raccoons. In a very limited captive study, Ough (1982) observed daily six wild-trapped male raccoons housed in individual pens from July 1978 to April 1979. All raccoons urinated and defecated, but only two individuals displayed scent-marking behaviors. These two individuals anal rubbed, which was sometimes accompanied by fecal smearing, and one of the two was observed to neck rub ten times. During the study, raccoons were also individually placed, sequentially, in a common chamber where all individuals urinated and four defecated. Due to the repetition of marking or urinating at

the same sites by different individuals in the common chamber, Ough (1982) suggested over-marking may be occurring.

In order for these sources of odors: neck rubbing, anal rubbing, urination, and defecation, to be used for communication, it is necessary that raccoons in the wild come into contact with odors from other individuals. Adult male raccoons have been found to spend from 20-30% (Gehrt *et al.* 2008) to as much as 94% of their time with fellow male coalition members (Pitt *et al.* 2008). However, they are only found in the presence of females during the breeding season. While not in direct contact, raccoons may communicate through the use of communal latrines. These were first observed by Davis (1907) who noted that raccoons in groups as large as seven exclusively urinated and defecated in only one or two corners of their enclosure. In the wild, communal latrines have been documented by Stains (1956) and Page *et al*. (1998) who observed three raccoons using one latrine site and found latrines were most commonly located on logs or stumps, and at the base or in the forks of trees. Though no functional significance has yet been found for these latrines (Gehrt 2003), intraspecific olfactory communication has been postulated as the most probable explanation (Ough 1982).

By studying patterns of scent-marking, we can gain important insights that move us toward understanding what types of information may be shared through these behaviors. Observing the patterns of responses of one individual to the scent-marks of other group members, for example sniffing, licking, or over-marking, can suggest that biological odors are used for competitive marking, territorial behavior, mate attraction, the formation of group scent, or as a means to identify individuals. Sniffing and licking

are common responses to scent-marks and are considered investigatory behaviors that provide information to the receiver about aspects of the sender's identity, sex, or physiological state. Licking specifically involves the transference of chemicals to the main olfactory system and possibly also the vomeronasal organ. The vomeronasal organ has been found to process sexual information (Estes 1972; Wysocki 1979), and the main olfactory system is important in making fine-grained discriminations such as those that may underlie individual or kin recognition (Johnston and Rasmussen 1984; Petrulis *et al.* 1999).

Another common response to scent-marking is "over-marking." Over-marking occurs when one individual places its scent-mark on top of, touching, or adjacent to the scent-mark of another individual, usually a conspecific (Ferkin and Pierce 2007). Johnston *et al*. (1994) outlined three scenarios for what might happen when over-marking occurs: scent blending, communal posting of chemical messages (a.k.a. a chemical bulletin board), and scent masking. Each of these scenarios assumes different functions for marking, with the first two being non-competitive. In scent blending, group odors are formed through the blending of individual odors. In the bulletin board scenario individual odors remain distinct, even when marked over, and are posted in a common area, becoming accessible to all conspecifics in a population. These odors may be posted for a variety of reasons including, but not limited to, mate attraction or to indicate current residency in an area. A prediction of the non-competitive scenarios is that scent-marks will be placed along well-traveled paths by many individuals. A specific prediction of mate attraction through the use of a chemical bulletin board is that individuals will over-

mark the marks of opposite-sex conspecifics more than same-sex conspecifics (Ferkin and Pierce 2007). The third scenario is a competitive form of marking where a top scent-marker, seeking to gain territory, resources, or mate-choice advantage, may physically mask the presence of a previous scent-marker (Ferkin and Pierce 2007). A prediction of the competitive marking scenario is that individuals will over-mark same-sex conspecifics more than opposite-sex conspecifics and, likewise, will over-mark same-sex competitors more than same-sex non-competitors.

Other important scent-marking patterns can be related to dominance or individual activity patterns. Asa *et al*. (1985) found in wolves that dominant males scent-mark with urine more frequently than subordinates. Scent-marking frequency may also be merely an incidental behavior correlated with activity level such that more active individuals have more opportunity to scent-mark. Such a relationship between marking and activity was reported by Holmes (1990) in opossums. In any case, the pattern of scent-marking that a species or gender employs can be expected to be closely intertwined with its social structure, habitat use, or physiology, such that wide generalizations should not made about functions until that species' system has been studied in detail.

In order to increase our understanding of the role that chemical communication plays in the socio-spatial behavior of raccoons, I conducted a study of their general scent-marking patterns with the following two objectives: 1) to determine how, where, and when raccoons scent-mark and respond to the scent-marks of conspecifics, and 2) to determine if raccoons deposit and respond to scent-marks in non-random patterns. I hypothesized that my subjects would scent-mark using urine, feces, anogenital rubs, face

rubs, and neck rubs. I predicted they would scent-mark, urinate, and defecate in communal latrines, and would respond to the scent-marks of conspecifics through investigation and over-marking. I also hypothesized that dominance rank and activity level would influence patterns of scent-marking and predicted that scent-marking frequency would positively correlate with these two factors.

Methods

Subjects

In 1997, the Henry Doorly Zoo in Omaha, NE locally obtained 4 litters of wild-born juvenile raccoons and raised them communally in captivity. There are currently five individuals remaining, 2 males and 3 females of unknown relatedness (Table 1). All raccoons are 10 years of age, born the same spring. I do not have current weights for individuals or familial relationships because the zoo did not have records of these. Subjects have been housed in the zoo's "Kingdoms of the Night" mixed-species display since 2004. The exhibit has a reversed 12:12 light cycle; the lights come on at 21:00 hours and turn off at 09:00 hours. However, ambient lighting in the display allows visitors to observe the animals in the 'dark' during their most active period. The raccoons' main diet of Hill's Feline Science Diet® k/d, fresh fruit and boiled meat is provided in their holding cage. The raccoons are moved into this cage everyday at 18:00 hours for their "daytime" denning.

Table 1. Subjects

Raccoon ID Number	Description
1	Small Female
2	Larger Male
3	Male
4	Largest Female
5	Large Female

Housing

The Henry Doorly Zoo raccoons are housed in a mixed-species, naturalistic exhibit that includes terrestrial and aquatic features. Five large logs and a man-made tree are attached to a rock ledge which is 15.25 m x 1 m with 2.5 m of vertical climbing space. This ledge is situated above a 41,000 gallon fresh-water aquarium (Figures 1 and 2). Interaction among the terrestrial and aquatic species in this exhibit (American alligators, alligator snapping turtles, several other species of turtles and fish, and raccoons) was common. In the display, the aquarium was the only source of water and a timed dispenser distributed fish pellets hourly to all the display inhabitants. The aquatic species were also fed fresh fish daily. However, the raccoons often grabbed the fish out of the water before the alligators and turtles could get to it. The terrestrial portion of the exhibit was hosed down with water daily before the raccoons were given access and keepers entered the fresh water aquarium twice weekly to clean the glass. Due to its position above the aquarium, the terrestrial habitat could not be cleaned with soap, or any odor removers except for the occasional bleaching of the latrine areas.

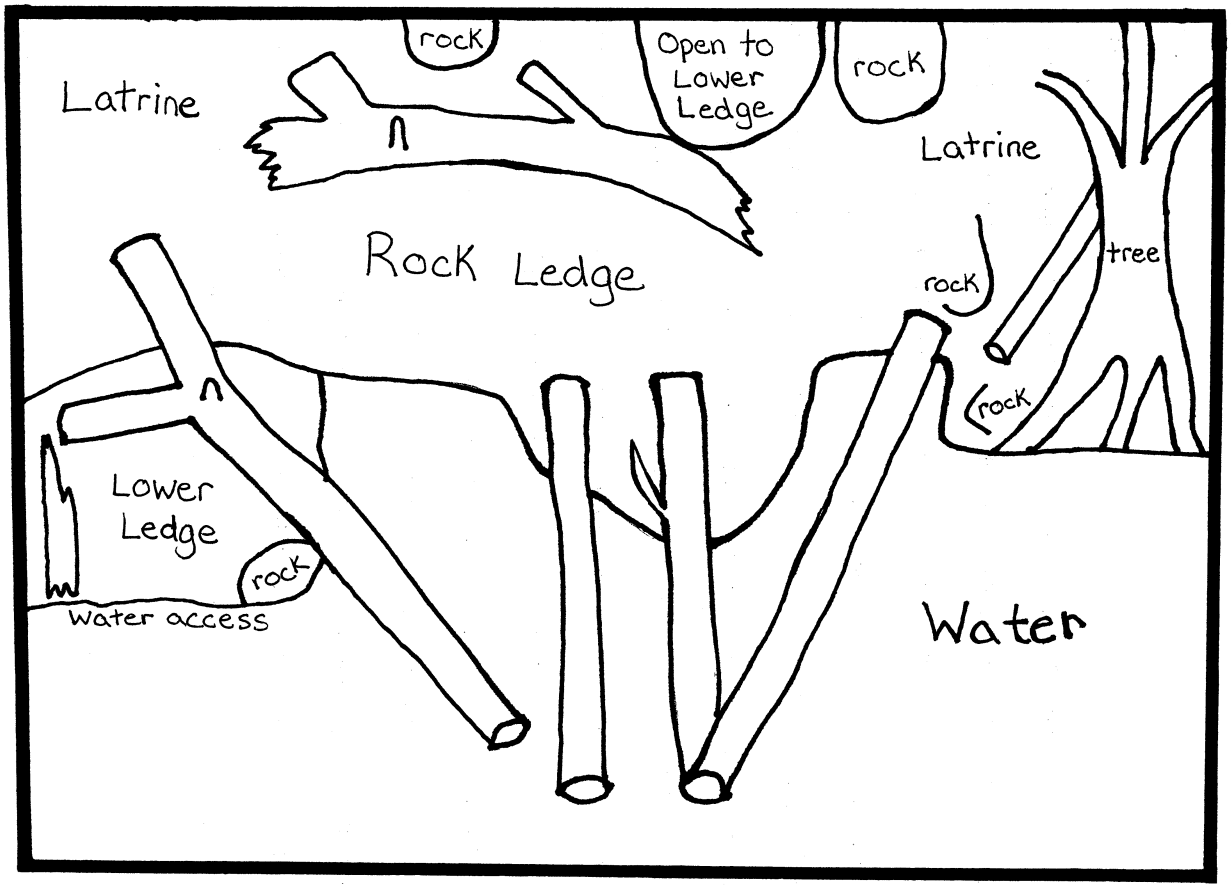

Figure 1. A birds-eye map of the raccoon enclosure at the Henry Doorly Zoo as seen from the observer's position.

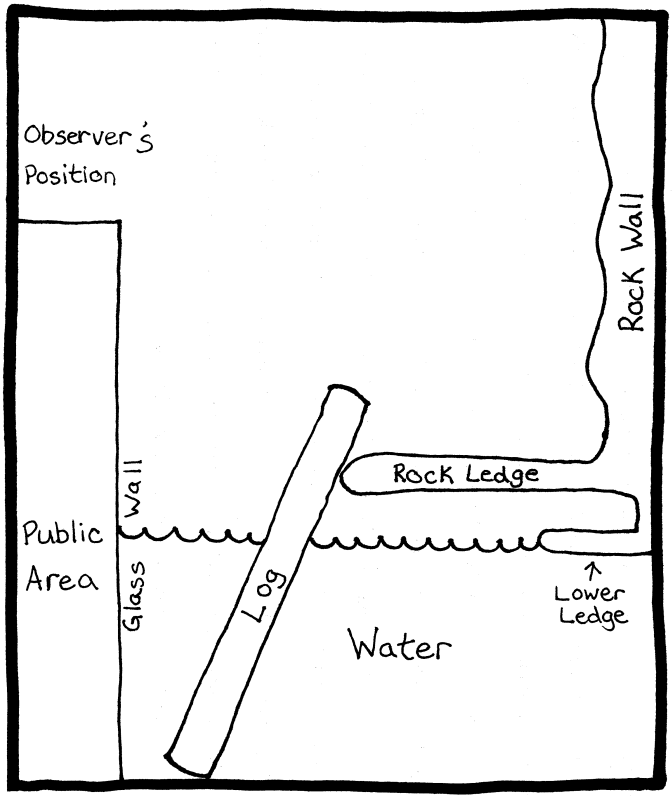

Figure 2. Side view of the center of the raccoon enclosure at the Henry Doorly Zoo. Only when subjects went down to the lower rock ledge, below the main ledge, were they not visible to the observer. The lower ledge was continuous along the back wall and connected to the lower ledge on the left side of the enclosure. Subjects on the lower ledge on the left side of the enclosure were visible to the observer (Figure 1).

Behavioral Observations

Sitting above the enclosure and looking down into it, I observed the subjects for a total of 78.6 hours; I did not manipulate their enclosure or daily routine in any way. Observations were made over six weeks for an average of four hours a day between 10:00 and 16:00 hours. Initially I observed subjects with focal individual sampling; however, due to the relative inactivity of some subjects I changed observation methods to instantaneous scan sampling after the first day of observation. Only data collected using

instantaneous scan sampling were used for data analyses. I documented the behavior of each individual every 30 seconds and at five minute intervals. I recorded the general activity level of all individuals as well as all behaviors that could be associated with scent-marking. I used the following non-mutually exclusive categories of behaviors: Active, Not Active, Not Visible, Grooming, Anogenital Rub, Neck Rub, Face Rub, Urination, and Defecation (Table 2). Anogenital rubbing may also include fecal and urine marking; however, it was not possible for me to determine when these two behaviors occurred in conjunction with the anogenital rubbing.

Table 2. Definition of Behavioral Categories

Active	Standing on all fours; includes walking, climbing and foraging
Not Active	Sitting or lying, includes moving head but not body
Not Visible	Subject climbed down to lower rock ledge where not visible to observer
Grooming	Self or mutual grooming with the teeth or tongue, considered a non-active behavior
Anogenital Rub	Squatting over an object and rubbing the rear-end front to back over the object repeatedly; similar to the definition of anal rubbing by Ough (1982).
Neck Rub	Rubbing back of neck on an object with head down towards the ground
Face Rub	Rubbing object from nose to check bones
Urination	Squatting posture over rock ledge or water and releasing urine
Defecation	Squatting posture over rock ledge with deposition of feces
Fight	Aggressive interaction involving growling or biting
Displacement	One individual pushing another one out of a preferred sleeping spot
Investigation	Sniffing or licking an object or area where group individuals routinely scent-marked; presence of odors at those sites is assumed
Over-mark	Marks made sequentially on the same object within a 15 minute time period. (Since I had no information on how long a mark could last in the environment or what previous odors might be present on an object, I used a very conservative measure so I could know with 100% accuracy that an individual was interacting with a freshly placed mark.)

I also recorded spontaneous agonistic interactions when they occurred, including fights and displacements (Table 2). Winners of fights or displacements were defined as the individual that was not displaced or chased off at the end of the encounter. These data were then used to create a dominance matrix.

I also recorded the location within the enclosure of all behaviors observed during the last 40 hours of my observations. In order to do this I gave each rock, log, latrine site and tree branch a numerical code and recorded that code with the behaviors as they occurred. Latrine sites were defined as areas where there was a consistent accumulation of urine and feces from multiple individuals at a higher density than in any other area.

Analysis

Patterns of Over-marking

A measure of over-marking frequency was obtained by counting marks made sequentially on the same object within a 15 minute time period (Table 2). If no scent-marking behavior had occurred at a particular area for 15 minutes, then the scent-mark of the first individual to mark that spot was termed the bottom mark and the scent-mark of the second individual to mark that spot was considered the top mark. If multiple sequential marks were deposited, the most recent mark was always considered the top mark, though marks between the first and last mark could be assigned both bottom and top mark status according to which individual the subject marked over and was over-marked by.

Based upon each individual's marking frequency, I calculated the number of male or female marks present in the environment during any 15-minute period. I then used

these data to predict the pattern of males and females over-marking same-sex and opposite-sex conspecifics. Predicted frequencies of marking were then compared to actual data. To calculate the number of male marks present, the sum rate of marking of all non-self males (m) was divided by the sum rate of marking of all non-self individuals (d). To calculate the number of female marks present, the sum rate of marking of all non-self females (f) was divided by the sum rate of marking of all non-self individuals (d). For example, if the two males both deposit 2 marks per hour and the three females all deposit 1 mark per hour, then the predicted frequency of a male over-marking another male would be m / d = 0.4 where m = 2 and d = (2+1+1+1). The predicted frequency of the same male over-marking a female would be f / d = 0.6, where f = (1+1+1) and d = (2+1+1+1).

Dominance Hierarchy

A dominance matrix was created using tallies of displacements and fights in which there was a clear winner or loser. I determined the degree and significance level of linearity for the dominance hierarchy using the software MatMan™ v 1.1 (Noldas 1998). In addition, I tallied the number of fights and displacements each individual was involved in.

Location of Scent-marks

A Chi-Square test was run to determine if the deposition of anogenital rubs and over-marks was significantly different from random. For the observed frequencies, I divided the enclosure into three zones, each of approximately the same area and containing the same number of possible places to deposit anogenital rubs. Then, I

recorded the number of anogenital rubs or over-marks deposited by each individual in each zone. If scent-marks were placed at random, I expected to find 33.3% of each individual's total anogenital rubs in each zone.

To further analyze the data, I also categorized all the marking sites in the enclosure as either a) near latrines, b) near water, or c) other and tallied each individual's marks per category. The latrines are highly-visited and highly-investigated areas by all members of the group. It is possible, then, to assume that a greater number of marks found near the latrines could indicate a benefit to individuals of marking in highly-traveled areas, or of marking on top of or near other individuals. The main water-access point for the raccoons was the large, low-lying rock platform on the left side of the enclosure (Figure 1). On this rock platform, aggressive encounters over fish, fish pellets, and access to the water were observed. It is possible, then, to assume that a greater number of marks found near the water is associated with marking near valuable resources. A greater number of marks found in other places suggests there is some spatial distribution of marks by individuals that does not fit either hypothesis.

Principal Components Analysis

The frequency of all behaviors recorded was tallied for each individual. I used a Principal Components Analysis (PCA) using SPSS® 17 to isolate which dependent variables accounted for the maximum amount of the variation in the data. The dependent variables included in this analysis were frequency of anogenital rubs, neck rubs, face rubs, investigation, urination, and defecation, gender, dominance rank, involvement in agonistic interactions, activity level, number of top marks, and number of bottom marks.

Next, Spearman correlations were run between high loading variables to find if there were significant relationships.

Results

Four principal components explained 100% of the variance among my twelve dependent variables. Those dependent variables with high loadings (0.80 or higher) for each principal component were identified and further examined in Spearman correlations. The first component, with an eigenvalue of 5.16, accounted for 42.96% of the total variance and presented high loadings for anogenital rubs (0.94), gender (0.93), number of top marks (0.94), and number of bottom marks (0.88). The second component, with an eigenvalue of 3.55, accounted for 29.58% of the total variance and presented high loading for defecations (0.83) and activity level (0.82). The third component, with an eigenvalue of 2.24, accounted for 18.64% of the total variance and presented a high loading for face rubs (0.961). The fourth component, with an eigenvalue of 1.06, accounted for 8.82% of the total variance and did not present a high loading for any variables.

Objective 1: Determine how raccoons scent-marked and responded to the marks of conspecifics

I observed a total of 437 anogenital rubs, 9 face rubs, and 9 neck rubs (Table 3). All individuals were observed anogenital rubbing; only one male neck rubbed (raccoon 2) and only three individuals (raccoons 1, 2, and 5) face rubbed. The majority of face rubs (66.7%, n = 9) were performed by the largest female. Anogenital rubs were placed on logs, rocks, branches and roots (Figure 1). All males and females were observed to urinate and defecate in two designated communal latrine sites. Raccoons anogenital

rubbed after urination or defecation between 9% and 36% of the time. No marking with urine was directly observed; however, one male habitually raised his rear leg and dragged his anogenital region when passing one centrally-located log near one of the latrines. This behavior is similar to what has been described as "male raised-leg urination," a recognized form of scent-marking in wolves and foxes (Kleiman 1966). However, in the case of this raccoon it was impossible to determine if any urine was being deposited with the raised-leg behavior. I did not observe any clear marking with feces, but individuals defecated in two latrines as well as in other areas around the enclosure.

Table 3. Gender means of tallied scent-marks observed

Scent-mark	**Male**	**Female**
Anogenital Rub	153.5	43.3
Face Rub	1	2.3
Neck Rub	4.5	0

Subjects were observed responding to scent-marks with both investigation and over-marking. All individuals investigated odors at varying rates from 0.93 to 2.06 investigations per hour (male mean = 1.78/hr; female mean = 1.42/hr), but investigation rate was not significantly correlated with activity (Spearman $r = 0.8$, $p = 0.10$, $n = 5$). Though investigation often coincided with scent-marking, investigation rate was also not significantly correlated with frequency of anogenital rubbing, urination, or defecation (Spearman $r = 0.4$, -0.67, and 0.1, $p = 0.51$, 0.22, and 0.87 respectively, $n = 5$).

I observed thirty-one over-marks where one individual clearly sniffed a recently marked object and placed its mark on top of the previous one. On average, males deposited more over-marks than did females (male mean = 14; female mean = 6) which is

consistent with their overall higher rate of marking; the two males (raccoons 2 and 3) and one of the large females (raccoon 5) over-marked with the greatest frequency.

In comparing the predicted number of marks deposited over the marks of same-sex and opposite-sex conspecifics, only one individual, raccoon 2, deviated from the predicted values (Table 4).

Table 4. Predicted and observed values presented as proportion of total over-marks.

	Over-marks over Males		Over-marks over Females	
	Predicted	Observed	Predicted	Observed
Raccoon 1	0.76	0.6	0.24	0.4
Raccoon 2	0.53	0.24*	0.47	0.76*
Raccoon 3	0.55	0.64	0.45	0.36
Raccoon 4	0.77	1	0.23	0
Raccoon 5	0.81	1	0.19	0

* Raccoon 2 deviated from the predicted pattern by placing more over-marks over females and fewer over males than would have been expected if over-marks were being placed randomly over the marks present in the environment.

The frequency of anogenital rubbing was significantly positively correlated with both the frequency of top marking and bottom marking (Spearman: top marking $r = 0.9$, $p = 0.04$, $n = 5$; bottom marking $r = 0.9$, $p = 0.04$, $n = 5$) (Figure 3). Bottom and top marking were also positively correlated (Spearman $r = 1$, $p < 0.01$, $n = 5$) meaning that an individual that deposited more top-marks also deposited more bottom-marks. The frequency of top marking was not correlated with any other behavior.

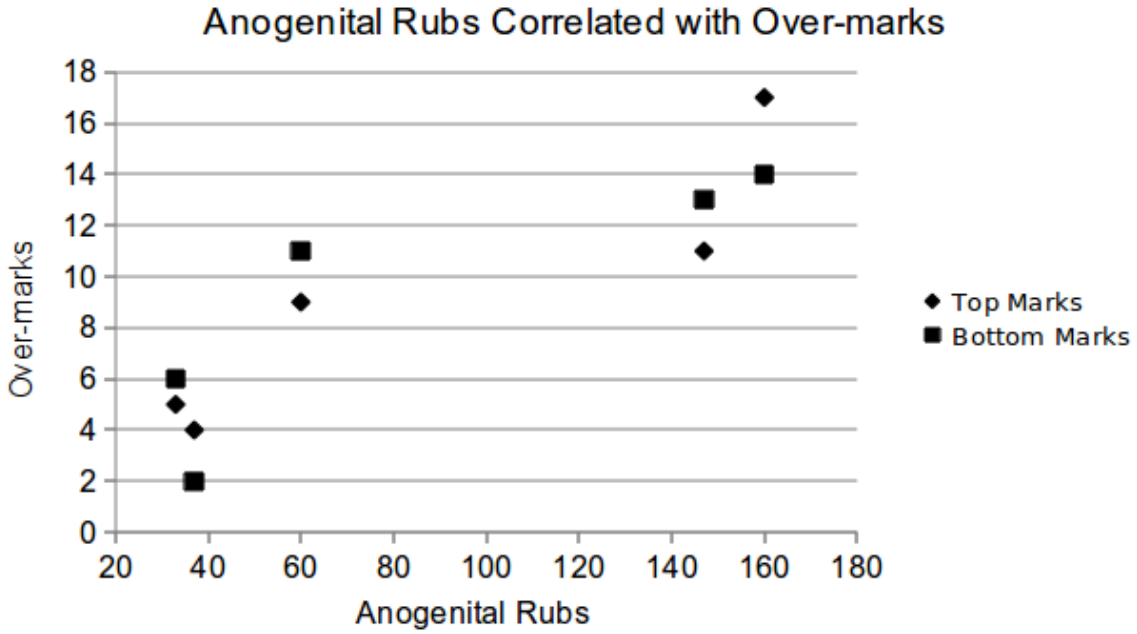

Figure 3. The frequency of anogenital rubbing was significantly positively correlated with both the frequency of top marking and bottom marking (Spearman: top marking r = 0.9, p = 0.04, n = 5; bottom marking r = 0.9, p = 0.04, n = 5).

Objective 2: Determine if raccoons deposited and responded to scent-marks in non-random patterns

The dominance matrix, based on the winners of fights and displacements, assigned raccoon 4 as dominant followed by raccoon 3, 2, 5, and 1 with one inconsistent relationship between raccoons 3 and 5. These ranks were not significantly correlated with any scent-marking behaviors. However, this dominance hierarchy was not significantly linear (Landau's linearity index: h = 0.55, p = 0.47). Therefore the hierarchy based on the dominance matrix for this group is problematic.

Scent-marking patterns were related to gender, with males scent-marking at more than twice the rate of females (Figure 4). No correlation between the frequency of anogenital rubbing and activity level (Spearman's r = -0.1, p = 0.87, n = 5) was found, but

involvement in agonistic interactions was positively correlated with activity level (Spearman's r = 0.9, p = 0.04, n = 5) (Figure 5).

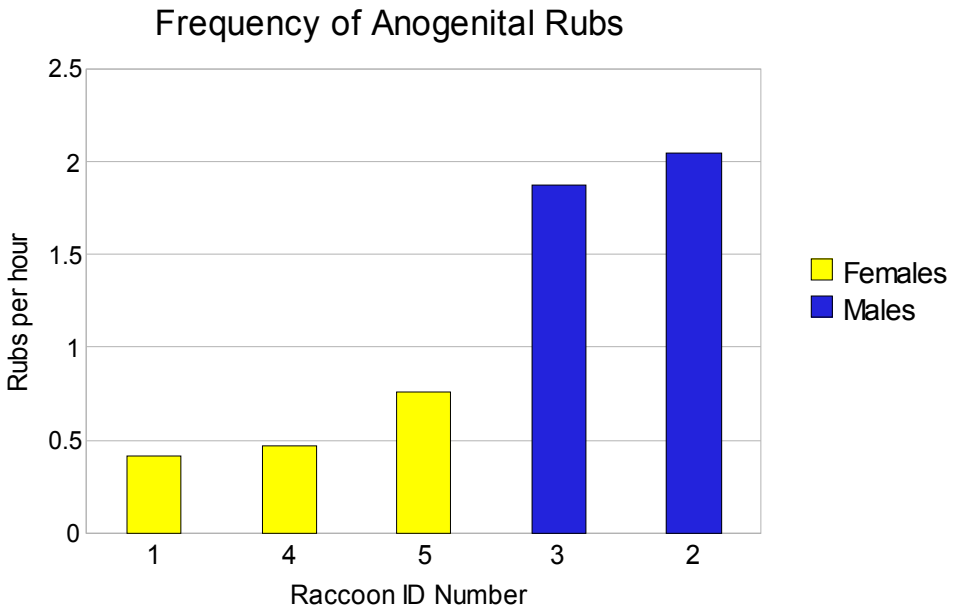

Figure 4. Females (light bars) anogenital rubbed at less than half the rate of males (dark bars) (n = 5).

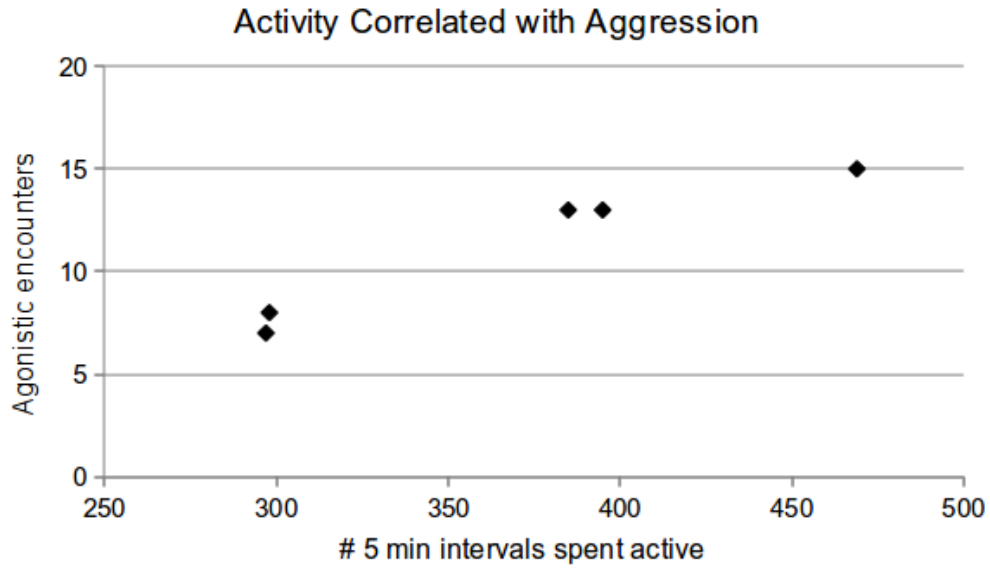

Figure 5. Involvement in agonistic encounters, including fights and displacements, was positively correlated with activity level (Spearman r = 0.9, p = 0.04, n = 5).

The subjects distributed their anogenital rubs non-randomly in the enclosure (Chi-Square $X^2 = 35.27$, df = 8, $p < 0.001$). The over-mark data did not have a sufficient number of data points for a Chi-Square test to be run.

For the group as a whole, 25.6 % of anogenital rubs were placed near water, 50% were placed near the latrines, and 24.4% were placed in other areas (n = 234). Of the total over-marks, 38.7% were placed near water, 51.6% were placed near the latrines, and 9.7% were placed in other places (Figure 6).

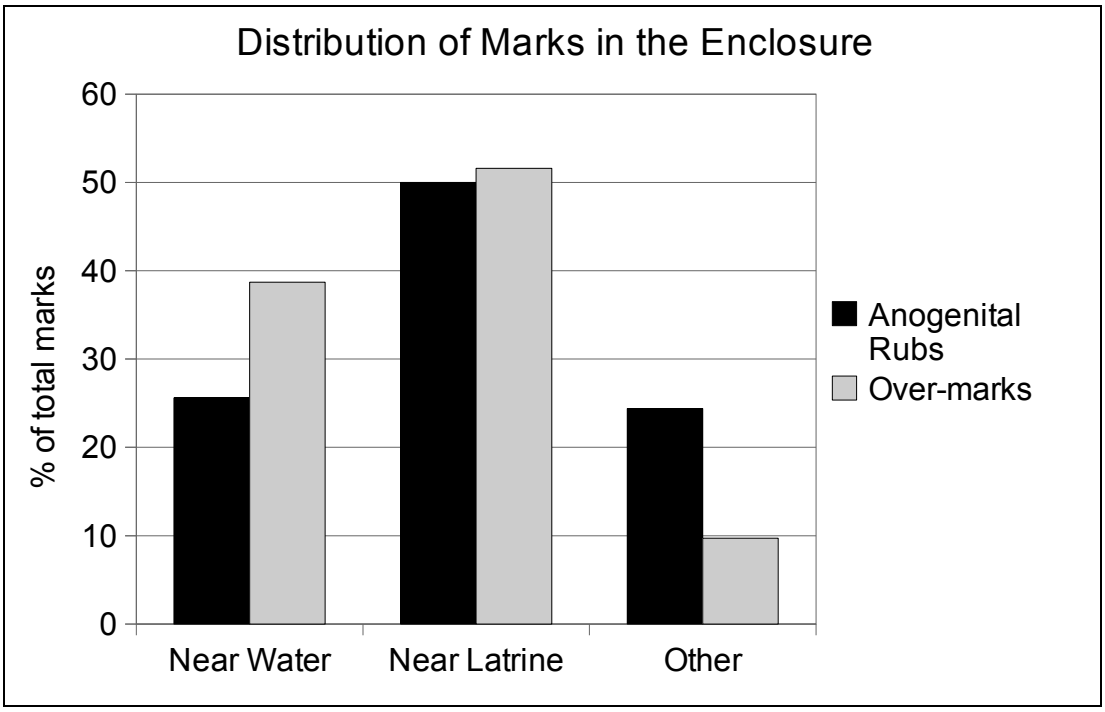

Figure 6. For the group as a whole, 25.6 % of anogenital rubs (black bars) were placed near water, 50% were placed near the latrines, and 24.4% were placed in other areas (n = 234). Of the total over-marks (gray bars), 38.7% were placed near water, 51.6% were placed near the latrines, and 9.7% were placed in other places.

Discussion

Though individuals showed a wide range of scent-marking behaviors, anogenital rubbing was by far the most common method of scent-marking observed in this captive population, a finding that is consistent with that of Ough (1982). It is not clear, however, what comprised the mark left from anogenital rubs. Ough (1982) reported that urination often occurred in conjunction with neck rubbing or anogenital rubbing whereas defecation was rarely associated with these activities. Holmes (1990) found that anogenital rubbing was significantly correlated with urination and defecation in male and female opossums. In my study, raccoons anogenital rubbed after urination or defecation between 9% and 36% of the time, suggesting that in addition to anal sac secretions, urine and feces also may have been deposited. However, even outside of elimination, it is still possible that urine and feces were deposited along with anal sac secretions during anogenital rubbing. The most frequently used spots for scent-marking in the enclosure were clearly discolored brown from a buildup of feces, urine or gland secretions. Clearly, future studies are needed to determine what is really being deposited with these scent-marking behaviors and at what frequency multiple odors are deposited. As this study was the first to systematically quantify raccoon scent-marking patterns, there are no studies of wild raccoons with which to compare these results.

The use of communal latrines and frequent investigation of the latrine areas strongly suggest that urine and feces are possible social odors. The use of feces for chemo-communication has also been suggested in ringtails, *Bassariscus astutus*, another procyonid, because they have been observed to defecate in latrines and leave single feces

on conspicuous substrates (Barja and List 2006). The use of what I suggested was raised-leg urination by one male raccoon may be an example of urine marking. In other species, such as wolves, raised-leg urination is only practiced by the most dominant individuals in a group (Asa *et al.* 1985). However, due to my group's lack of a dominance hierarchy, it is not clear what this behavior may be related to in raccoons. Raccoon social structure is sufficiently different from wolves, and so it is possible that raised-leg urine marking may serve a different function.

Raccoons were also found to respond to the marks of conspecifics through over-marking. Patterns of over-marking did not meet the criteria for a competitive marking hypothesis because most individuals simply over-marked whatever marks were available, irrespective of gender. Only one individual, raccoon 2, deviated from the predicted proportions and over-marked over females more frequently than would have been expected by chance, considering the number of female marks available for over-marking. As a group, the over-marking data best support a "scent blending" or "chemical bulletin board" hypothesis. It is not known, in the case of raccoons, whether scent-marks remain distinct or blend when layered. Raccoon 2 meets the criteria for a mate attraction hypothesis since it marked more frequently over opposite-sex conspecifics. However, because of the small sample size and the fact that only one male behaved in this manner, no definitive conclusions are possible.

The correlation between anogenital rubbing and over-marking indicated that individuals over-marked in proportion to their overall frequency of marking. In addition, since number of top marks and number of bottom marks were tightly correlated, it is

unlikely that a scent-marker benefits significantly from being the top-marker. Accordingly, individuals did not seem to make an effort to keep their marks on top. This draws into question whether the behaviors observed were even purposeful over-marks or just chance overlaps due to the limited locations for marking and the relative frequencies of marking for the five subjects. Similar results were found in a study of female meadow voles, *Microtus pennsylvanicus*; females in post-partum estrous were more likely to have their scent-marks over-marked by males only because females in this condition mark more frequently (D. Lee, pers comm., unpublished data).

In addition, raccoons deposited their anogenital rubs non-randomly throughout the enclosure. Over 50% of marking activities were concentrated near the latrines. The latrine areas can be characterized as easily accessible spots along well-traveled paths, consistent with a non-competitive scenario of scent blending or a chemical bulletin board. As scent blending is seen primarily in colonial species (Johnston *et al*. 1994), the most likely functional explanation for the patterns seen in raccoons is a chemical bulletin board. In this scenario, individuals use scent-marks to indicate their presence in an area; scent-marks are individually distinctive, and there is no benefit to top or bottom scent-markers in over-marks (Wolff *et al.* 2002 as cited in Ferkin and Pierce 2007). The over-marking results already discussed are also consistent with these predictions for non-competitive over-marking on a chemical bulletin board. Fritzell (1978) studied the patterns of raccoon home ranges, and from his data suggested that scent stations were of primary importance for male and female raccoons to advertise their presence in an area. Considering the raccoon's use of overlapping home ranges and shared den sites, a

message that indicated an individual's presence in an area would be ecologically advantageous for both senders and receivers.

Raccoons were also found to deposit and respond to scent-marks non-randomly. Males marked at over twice the rate of females but this difference could not be explained through dominance or activity level. This pattern is consistent with wild observation of coyotes, *Canis latrans* (Wells and Bekoff 1981) and captive observations of the maned wolf, *Chrysocyon brachyurus* (Dietz 1984). However, in the maned-wolf study, scent-marking was correlated with activity because the males were more active than females, which Dietz (1984) suggested may have been related to the male's more prominent role in territorial defense. In my study, the two males were more active than the two larger females. However, the one small female was extremely active yet scent-marked little. Further studies with larger sample sizes and, preferably, wild observations are necessary to further understand the correlates of male scent-marking in raccoons.

In the current study of raccoons, activity level was correlated with participation in agonistic interactions, suggesting that matrices based on agonistic interactions may not be the best measure of dominance for raccoons. Furthermore, winners of fights are hard to determine and agonistic interactions among individuals were not sufficiently consistent to yield a significantly linear hierarchy. Another method that has been used in an attempt to measure dominance in raccoons is the observation of their submission and aggression behaviors in forced dyadic interactions (Barash 1974). When immediate submission was observed, Barash (1974) assumed that the raccoons had interacted in the past and that the behavior exhibited during the experiment indicated a previously established dominance

rank. If aggression and fighting occurred, Barash (1974) assumed the two individuals did not have an established dominance relationship. Using similar observation of submission and aggression, Ough (1979 as cited in Ough 1982) noted that only the male raccoons that he had determined to be dominant individuals were seen scent-marking in his study. Conversely, all individuals in my study were observed to scent-mark. In a captive study of coyotes, Wells and Bekoff (1981) found likewise that all pack members scent-marked but dominant males marked more often than dominant females. This finding is consistent with the gender skew observed in my study but the group I observed did not have clear dominant individuals. Considering the natural history of raccoons, linear hierarchies or even consistently dominant individuals may not exist. Individuals sharing overlapping home ranges may simply have dyadic dominance relationships with all other individuals but no consistent relationships amongst all individuals. Dyadic dominance relationships were really what Ough (1982) and Barash (1974) were measuring in their dominance studies. Consequently, correlating scent-marking frequencies with the results of these dyadic relationships may have been inappropriate.

However, not all behavioral studies, especially non-primate studies, consider linearity in their dominance hierarchies. The Holmes (1990) study of captive opossums, for example, used a dominance matrix to determine dominance relationships without testing for linearity. I found two correlates of dominance in my data consistent with that study: dominant females face rubbed the most, and the frequency of male scent-marking was not correlated with dominance or involvement in agonistic interactions. It is

interesting that these results are similar, but without linear hierarchies or clear dominant individuals, these results may be meaningless.

When attempting to explain the patterns of scent-marking observed, it may also be important to note that subjects of this study were only exposed to scents of known individuals from their group. In addition, no individuals immigrated into or emigrated out of the group during the study. In the wild, individuals encounter unfamiliar individuals more frequently and coalition membership changes over time, both of which can affect scent-marking patterns. For example, in coyotes, scent-marking patterns have been found to vary considerably based on the size of the group (Wells and Bekoff 1981). According to MacDonald (1985), the subtleties of an individual's status and social relationships with each of its neighbors are likely to affect its inclination to mark in a given way under given circumstances. Therefore, though all the circumstances of this study may not have been analogous to the wild, this study provides a snapshot of scent-marking patterns in one captive group of raccoons with a depth of detail that would be impossible to capture in the wild.

Relatively little is known about how carnivores use social odors, and with the exception of the opportunistic observations of Ough (1982), nothing is known about raccoons specifically (MacDonald 1985). My study, which is the first to quantify and statistically analyze the scent-marking patterns of raccoons living in a social group, greatly advances our understanding of social odors in this species. Scent-marking is clearly a common and important behavior in raccoons and more studies are necessary to test the hypotheses presented here about the use of scent-marks for chemo-

communication. In my study, patterns of marking best supported the predictions of a chemical bulletin board hypothesis wherein the main function of marks is to advertise an individual's presence in an area. Further support for this hypothesis could be gained through the investigation of individually distinct odors in raccoons. Additionally, determining whether raccoons are able to discriminate individual identities from the scent-marks left on chemical bulletin boards would go a long way in explaining patterns of scent-marking and the possible functions of biological odors in this species.

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Chapter 2: Discrimination of Individual Odors by the Raccoon

Introduction

The concept of chemical communication suggests that an individual's urine, feces, and/or scent-marks may contain information about the individual such as species, age, sex, reproductive condition, health status, or individual identity (Halpin 1980). The most specific kind of information found in odors is identity, which is only communicated through individual odors, or odors of sufficient complexity to be individually distinctive.

The ability to discriminate individual odors has been found in many mammalian species including, but not limited to, the Mongolian gerbil, *Meriones unguiculatus* (Halpin 1974), golden hamsters, *Mesocricetus auratus* (Johnston *et al*. 1993), mice, *Mus musculus* (Bowers and Alexander 1967), rabbits, *Oryctolagus cuniculus* (Mykytowycz 1972), pronghorn antelope, *Antilocapra americana* (Müller-Schwarze 1974), ring-tailed lemurs, *Lemur catta* (Mertl 1975, Palagi and Dapporto 2006), African dwarf mongooses, *Helogale undulata rufula* (Rasa 1973), wolves, *Lupus lupus* and dogs, *Canis familiaris* (Brown and Johnston 1982). In these species and others, the sources of discriminable odors are urine, feces, anal gland secretions, and secretions from many other specialized exocrine glands (Halpin 1980). For example, Halpin (1974) demonstrated that Mongolian gerbils can distinguish individual odor differences in the urine odors, ventral gland odors, and whole body odors of conspecifics. Similar results were obtained with anal gland secretions in mongooses where individual differences were based on the bacterial flora residing in the anal glands (Rasa 1973; Gorman 1976). In golden hamsters, Johnston *et al*. (1993) demonstrated the presence of odors sufficient for

individual discrimination in flank and ear gland secretions, vaginal secretions, urine and feces.

The importance of individual odors lies in their effect on the social behaviors of animals, and their role in social organization. Wilson (1970), for example, predicted that a critical difference between invertebrates and vertebrate societies was likely to be that in the latter, individually-distinctive odors would allow for individual recognition. This is in contrast to invertebrate societies (e.g. social insects) where recognition is usually between own group members versus non-group members, and not between individuals.

Individual odors can have a number of different functions. For example, individual odors can allow an individual to discriminate among members of its own social group (Hare 1992) and between neighbors and strangers (Harris and Murie 1982; Murdock and Randall 2001). Additionally, individual odors can play a role in kin discrimination (Paz y Miño C. and Tang-Martínez 1999; Tang-Martínez 2001; Mateo 2004) and in mate recognition (Newman and Halpin 1988). When influenced by the MHC complex, individual odors may also play an important role in mate choice decisions, allowing individuals to avoid inbreeding or to choose genetically advantageous mates (Tregenza and Wedell 2000; Yamazaki and Beauchamp 2007). Lastly, individual odors may facilitate the formation of dominance hierarchies and establishment and maintenance of territories. When conspecifics attribute odors to certain individuals in a population and that identity can be remembered through time, then individual recognition can occur. Based on individual recognition and the memory of previous interactions, an individual may respond to another individual differently and may form consistent

relationships with a number of different individuals. Determining the importance of individual odors in a species first requires an understanding of the chemical nature of the odors and the ability of conspecifics to detect the necessary differences.

Over the past thirty-five years, the habituation-discrimination paradigm has been one of the most widely used techniques to investigate the occurrence of individual odors and the ability of conspecifics to discriminate individual differences in odors (Tang-Martínez and Bixler 2009). In this technique a subject is first habituated to the odor of one conspecific and then presented with the odor of a second conspecific concurrently with the odor to which it just habituated. Habituation is defined as a decrease in investigation over time. The prediction is that if the test animal can discriminate individual differences, it will spend more time investigating the second, unfamiliar odor as compared to the familiar odor. If the odors are not discriminated, which indicates that the animal cannot or chooses not to discriminate between them, then the test animal should respond randomly to the two odors. The model tests only for discrimination between two odor samples and says nothing about preference or recognition of an individual *per se* (Halpin 1986).

A variation of this technique, which is used frequently in psychology research, is familiarization-discrimination. In this technique, the subject is given time to become familiar with the first odor source and then the familiar odor is presented with an unfamiliar odor. The prediction is the same: if discrimination occurs, the subject should spend more time with the unfamiliar odor. Familiarization has been shown to effectively increase a subject's ability to discriminate between two odors (Jehl *et al*. 1995); it is

considered the learning of an odor which results in neural changes at the level of the olfactory bulb. These changes refine the pattern of activity in the main olfactory bulb in response to the learned odor, enhancing its discrimination from those of similar odors (Brennan and Kendrick 2006). Familiarization tests have been used to demonstrate discrimination of odors in a variety of species from discriminating unfamiliar aromatic compounds in humans (Jehl *et al*. 1995), to social recognition in rats, *Rattus norvegicus* (Engelmann *et al*. 1995), and discrimination of conspecific urine in dogs (Lisberg and Snowdon 2009).

Though studies on individual odors have been done in other carnivores, MacDonald (1985) notes that knowledge of social odors among the Procyonidae is even more fragmentary than among other carnivore families. More than 20 years later that statement is still true. Recently work has been done on fecal marking in the ringtail, *Bassariscus astutus* (Barja and Lista 2006); this research suggests that fecal latrines and single feces are used for communication, but more work is needed to determine the functional significance of this type of marking. Moreover, in raccoons, the most common North American procyonid, almost nothing is known about the function of social odors or an individual's ability to discriminate and gain information from social odors.

There is increasing evidence that social odors may play a role in the spatial distribution and social behavior of raccoons. Raccoons have been found to form social relationships both in captive studies and in the wild. In captive studies, raccoons display scent-marking behaviors, deposit social odors with anogenital rubs, neck rubs, urine and feces, and utilize communal latrines (Davis 1907; Ough 1982; Kent 2009: Chapter 1). In

addition, raccoons investigate the marks of conspecifics at a high rate and do not show signs of competitive marking (Kent 2009: Chapter 1). In the wild, males and females have overlapping home ranges (Stuewer 1943; Kamler and Gipson 2003; Walker and Sunquist 1997; Gehrt and Fritzell 1997). Moreover, at high and low densities males form coalitions, defend a territory communally, and share resources, dens and possibly access to females (Gehrt and Fritzell 1998; Pitt *et al.* 2008). This is an unusual form of sociality for a male carnivore as it does not fit with any prevailing hypotheses to explain home range distribution. The large overlapping home ranges of males, sometimes as much as four times larger than the females' home ranges (Pitt *et al*. 2008; Gehrt and Fritzell 1997), have been found to be larger than predicted based solely on energetic requirements. They also are stable throughout seasons and, thus, not mating-dependent (Gehrt and Fritzell 1997). In addition, coalition formation between males is not directly dependent on the aggregation of females (Pitt *et al*. 2008), nor is it regulated by kin selection as groups are not composed solely of close relatives (Gehrt *et al*. 2008). Therefore it appears that social interactions, which include scent-marking and olfactory communication, may be one of the most important factors regulating home range size and socio-spatial behavior in male raccoons.

The benefits to raccoons of having discriminable individual odors include the ability to recognize kin to avoid inbreeding, to recognize individual coalition partners as well as mating partners, to recognize non-coalition males when encountered in their home range, and to react appropriately to dominant individuals based on remembered

previous interactions. Possible sources of individual odors for raccoons include urine, feces, and anal sac secretions.

The objectives of this study were to determine if raccoons could discriminate individual differences in the odors of urine, feces or anal sac secretions using a familiarization-discrimination test derived from the habituation-discrimination paradigm. I predicted that raccoons would spend more time investigating unfamiliar odors as compared to familiar odors.

Methods

Test Subjects

Test subjects consisted of healthy, wild-trapped, sub-adult or adult male and female raccoons. A total of 23 animals were trapped from October 2008 to May 2009, 14 males and 9 females. Whenever possible, test subjects were trapped near the test site to reduce the stress of a novel environment. However, due to low rural population densities, only a limited number of raccoons could be obtained on-site and some had to be brought in from surrounding areas. Raccoons were aged by observation of tooth wear, body condition and the visibility of nipples or testicles in the breeding season. No lactating females or juveniles under six months of age were used as test subjects. From the 23 individuals trapped and held for a maximum of 72 hours, I obtained 10 nights of successful urine tests with 4 female and 6 male subjects, 8 nights of feces tests with 3 female and 5 male subjects, and 3 nights of anal secretion tests with 1 female and 2 male subjects. An individual's data were not used if the subject did not sniff the first box

during the familiarization phase, did not sniff both boxes during the discrimination phase, or escaped during the night.

Study Sites

Tests were conducted in large outdoor enclosures specifically designed for holding raccoons. These enclosures were located at wildlife rehabilitation facilities in Omaha, Nebraska and Shiloh, Illinois. The facility in Omaha had two enclosures available. The first enclosure was a 3.6 meter cube with a wood frame and was covered in 12 cm x 6 cm welded stainless mesh. The second enclosure was 3.6 m long, 1.5 m wide and 2.4 m tall. This enclosure had a metal frame and all sides were covered by 2 cm x 1 cm plastic-coated wire mesh. The enclosure in Shiloh, Illinois was 3 m long, 1.8 m wide and 2.4 m tall and consisted of a metal frame covered in chain-link fence. The tops of all the enclosures were covered with boards or plastic to provide shelter from the elements. Inside each enclosure was a basin of water, a den-like shelter, and food provided *ad libitum*.

All of the enclosures were frequently visited by local wildlife including, but not limited to, raccoons, opossums, skunks, and feral cats. During such visits the test subjects inside the enclosure retreated to the upper levels of the enclosure and avoided the visitors. Only once did a test subject (female) inside the enclosure respond aggressively towards an unfamiliar raccoon outside of the enclosure. Before and after each test, enclosures and all contents and surfaces within them were cleaned using a water, bleach, and dish soap solution. All feces and soiled bedding were removed prior to cleaning.

Test subjects were housed for a maximum of 72 hours in the enclosure. After testing was completed, subjects were released where they had been trapped.

Collection of Samples

Urine and feces were collected from anesthetized sub-adult and adult male raccoons trapped on the grounds of the St. Louis Zoo or in the surrounding urban park, Forest Park. These raccoons were trapped as part of the zoo's general raccoon tracking and management program. The raccoons I used as odor donors were of unknown relatedness and considered to have the same general diet and habitat. Urine was collected via cystocentesis, inserting a needle through the abdominal wall into the bladder, and feces were collected by inserting a fecal loop into the anus, or after natural elimination. Anal sacs were excised from freshly euthanized males who had been removed from the zoo grounds because they had become a nuisance. Samples were collected with the aid of the veterinarians and technicians at the St. Louis Zoo and frozen immediately after collection at -20°C until use. Four feces and one urine sample were also collected opportunistically from male test subjects at study sites in Omaha, Nebraska and Shiloh, Illinois. Raccoons were only tested with samples collected from a different state to control for the possibility of previous social experience with odor donors. In addition the odors presented to test subjects were randomized based on the age of the odor donor and age of the test subject.

Test Apparatus

The odors were presented to test subjects in a locked metal security box (4.75 cm H x 16.8 cm W x 17.8 cm D) with 5-mm holes drilled through all six sides at 2-cm intervals. Inside the box, a petri-dish, perforated with 80 2-mm holes on top and bottom, was suspended from the top of the box by a screw and nuts assemblage so that it was equidistant from all sides and was held in place securely to limit noise production when jostled (Figure 1). To prevent disease transmission, the design of the apparatus assured that the test subject had no direct contact with the odor sources. Odor sources were allowed to thaw outdoors for one hour before use. If the ambient temperature was too cold for natural thawing, the sample vials were dipped in a warm water bath until just above freezing. No effort beyond this was made to warm the odors above the outdoor ambient temperature. To 'load' the box, 15-20 drops of urine or ¼ teaspoon of moist feces was deposited on a 10-cm-diameter piece of filter paper that was then placed inside the petri-dish and locked inside the box. For the anal sacs, the sac was pierced with a straight pin so that all liquid secretion could be squeezed out onto the filter paper; then the filter paper containing the secretion along with the anal sac tissue was locked in the apparatus. It was necessary to use a metal box that locked with a key because raccoons are extremely dexterous and strong. They can manipulate any latch and tear through almost any material other than strong metal (Cole 1907). Before and after each use the test apparatus was cleaned with antibacterial dish soap, bleach and water and rinsed with 95% ethyl alcohol.

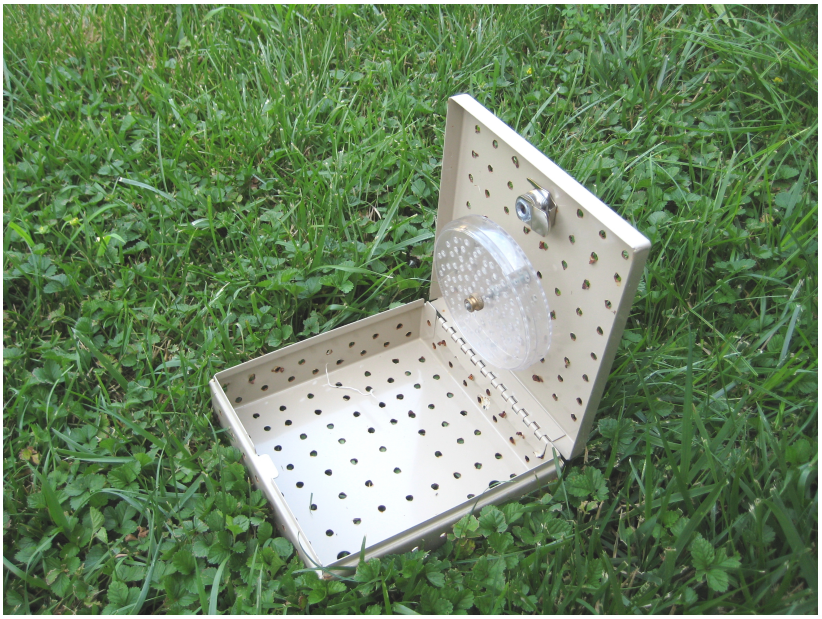


Figure 1. The odor box was comprised of a perforated metal security boxed with a perforated petri-dish containing the odor source suspended inside of it.

Familiarization-Discrimination Procedure

The familiarization-discrimination tests had two phases, a familiarization phase and a discrimination phase. During the familiarization phase, one 'loaded' odor box (containing odor from donor A) was placed in the raccoon's enclosure for two hours allowing ample time for investigation. Investigation was defined as sniffing the ground immediately next to the box, nose-box contact, or manipulation of the box. The first box was introduced within a half hour before or after dusk.

Immediately following the familiarization phase, a second box was added to the enclosure containing an unfamiliar odor (odor from donor B) of the same type, urine, feces or anal sac, as the first odor. The boxes were placed 60 cm apart in different locations from the first box. This controlled for familiarization with the location of the

box. The location of the two boxes and the odors used in each trial were also randomized to avoid side biases and to control for order effects. Since manipulation or direct contact with the box was rare during the familiarization phase, there was not any reason to believe the subjects had contaminated the box. Therefore, I used the same odor A box for both phases of the test. All odors used had the same exposure period between deposition on the filter paper and introduction to the test animal to avoid artificial differences in odor strength. The discrimination phase was recorded for six hours and after each trial, the filter papers, pipettes, and gloves were disposed of as biohazards.

Analysis

The familiarization-discrimination tests were recorded using infrared cameras and 8-hour VHS tapes. These tapes were then scored for frequency and duration of investigation of the odor boxes during the 2-hour familiarization and 6-hour discrimination phases. In initial observations, it was apparent that most investigation occurred during the first 2.5 hours of the discrimination phase. Therefore, 2.5 hours after the introduction of the second box was considered the discrimination phase for all subsequent tests. There was one exception: one subject did not leave the den until four hours into the discrimination phase. Since it is unlikely the individual could detect the odors while so far removed, we measured the 2.5 hours from the time of first emergence. I used the Wilcoxon matched-pairs test in SPSS® 17 to evaluate differences in time spent investigating odor A versus odor B in the discrimination phase. All analyses were two tailed and I employed exact tests as suggested by Mundry and Fischer (1998). A

significantly greater amount of time spent sniffing odor B during the discrimination phase was considered positive discrimination of the odors by the test subject, meaning that the odors are sufficiently different that the raccoon could discriminate between them.

Results

Familiarization

The familiarization-discrimination technique was used in this study because the test subjects did not show habituation to the first odor. The definition of habituation is a gradual decrease in the amount of time spent investigating (Halpin 1974, Halpin 1986). Therefore if habituation had occurred, the subject's investigation rate should have started high and tapered off during the two-hour period. This pattern did not occur. Though subjects showed interest in the first box, there was no consistent pattern of decreased or increased interest over the two-hour period (figure 2).

This pattern was true for both urine and feces. In the case of anal sac secretions, subjects showed little to no interest whatsoever in investigating the odor.

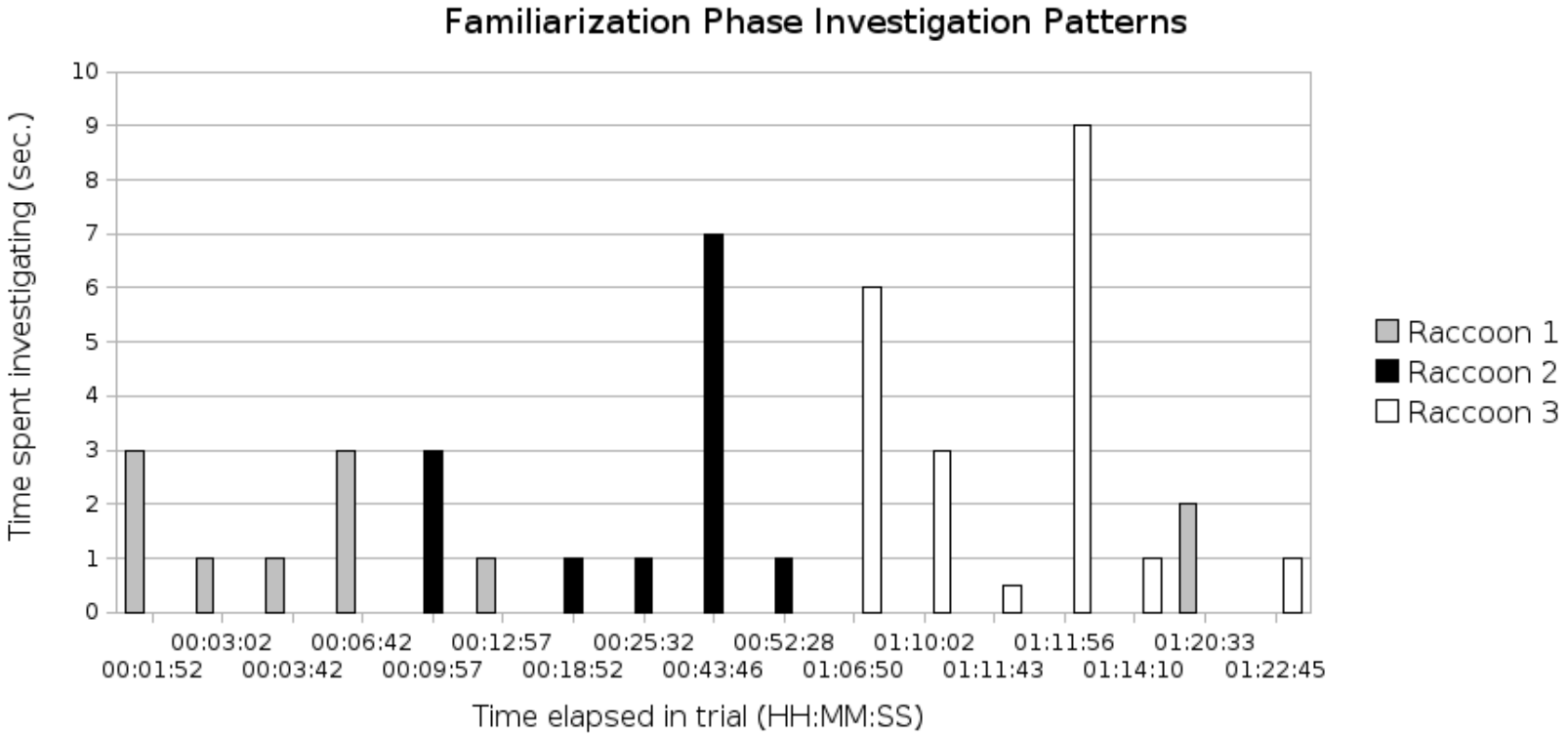
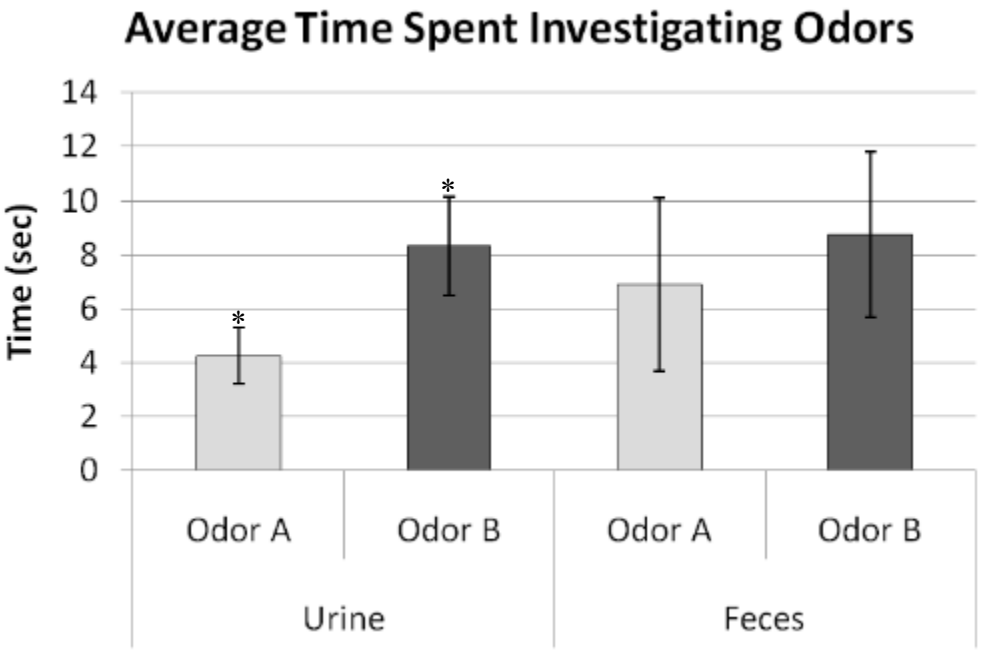


Figure 2. An example of investigation patterns for three representative individuals. Raccoons showed no consistent pattern of decreased or increased interest over the two-hour familiarization period. However, all raccoons did investigate the odor at some level during the two hour familiarization period.

Discrimination

The raccoons showed positive discrimination of the urine odors (Exact Wilcoxon signed ranks test p = 0.039, n = 10) (Figure 2). Overall, subjects spent on average twice as much time investigating the unfamiliar urine odor, odor B, as compared to the familiar urine odor, odor A, during the discrimination phase. There was no difference in the discrimination abilities of male and female subjects. Raccoons did not show discrimination of feces odors (Exact Wilcoxon signed ranks test p = 0.547, n = 8) (Figure 3). Because subjects showed little interest in anal sac odors and there was extreme variation in the subjects' responses, tests with anal sac secretion were stopped after only three subjects.



* p < 0.05

Figure 3. Raccoons showed significant discrimination between familiar (light bars) and unfamiliar (dark bars) urine odors (Exact Wilcoxon signed ranks two-tailed test p = 0.039, n = 10) but not feces (Exact Wilcoxon signed ranks two-tailed test p = 0.547, n = 8).

Discussion

The raccoons in this study exhibited clear discrimination between familiar and unfamiliar urine odors. Since sex was controlled for (i.e. all odor donors were male), and age and seasonal differences were randomized, this discriminatory response indicates the perception of differences that likely are based on genetic differences or other individual characteristics such as diet (Halpin 1986). Discrimination of individual differences in urine odors has been found using similar techniques in a wide variety of species including, but not limited to, the Mongolian gerbil (Halpin 1974), prairie voles, *Microtus ochrogaster* (Newman and Tang Halpin 1988), Norway rats, *Rattus norvegicus* (Carr *et al.* 1976), golden hamsters (Johnston *et al*. 1993), mice (Bowers and Alexander 1967), Eastern chipmunks, *Tamias striatus* (Keevin *et al*. 1981), sheep, *Ovis aries* (Baldwin and Meese 1977), wolves, and dogs (Brown and Johnston 1982).

Genetic differences in the urine odors of rodents have been linked to the MHC region of the genome (Singer *et al.* 1997; Yamazaki and Beauchamp 2007). Another explanation for genetically-linked odors is "odor-genes covariance" (Todrank and Heth 2003). This refers not to a direct relationship between particular genes and particular volatile compounds, but to a general relationship between individual genotypes and individual odors. According to these authors, the greater the genetic similarity between two individuals, the more similar their individual odors will be.

Genetic differences in odors allow for accurate kin discrimination, an ability which would be adaptive for raccoons in order to avoid inbreeding. In raccoons, sub-adult males disperse and females are philopatric and stay in their natal area.

Additionally, adult raccoons have stable home ranges throughout seasons (Gehrt *et al*. 2008). Therefore males have a high likelihood of encountering their daughters the following mating season. In fact, there is already some preliminary evidence that male raccoons have the ability to discriminate kin and may choose to form longer coalitions with closer relatives (Gehrt *et al*. 2008).

Genetic-based odor discrimination could also be adaptive because it allows for individual recognition. The benefits to raccoons of recognizing individuals include basing social relationships on past interactions for the establishment of dominance with coalition partners, recognizing mating partners, and recognizing non-coalition males when encountered in one's home range.

Differences in diet, such as protein intake, can also create individually discriminable differences in odors (Ferkin *et al*. 1997) though these differences are not necessary for individual discrimination to occur (Halpin 1986). In my study, though all odor donors came from a small geographic area, micro-habitat heterogeneity and individual preference may have allowed for differences in diet among individuals. Odor cues that rely on metabolic influences can be used for individual discrimination and even individual recognition if periodic updating of an individual's odor profile is possible (Hurst and Beynon 2004). It is then reasonable to suggest that densely populated, or highly social animals may be able to use metabolically-derived odor cues for communication. However, it is less reasonable to suggest that solitary or widely-dispersed animals would use such a mechanism.

Raccoons are a semi-social species and, as such, they are likely to only need to recognize a moderate number of individuals in their lifetime. These individuals include their mother, litter-mates, offspring, coalition partners, and mating partners. If their ability to discriminate individual urine odors relied upon metabolically-derived cues, raccoons would be able to recognize individuals within their home range such as coalition partners and mating partners because these odor signals could be updated frequently. However, for discrimination of unfamiliar kin from other unfamiliar conspecifics, some genetic basis of odors is necessary, regardless of which of the many hypothesized mechanisms raccoons may use for kin recognition. These genetic-based odors could allow raccoons to recognize half-siblings, sons, daughters, or unfamiliar intruders into their home ranges. For this reason, I suggest genetically-based odor cues would be more useful for chemical communication in raccoons. Such genetically-derived odors have been found in mice and rats, and it is likely that they may also exist in raccoons.

Raccoons did not exhibit the ability to discriminate individual differences in the odors of feces or anal sac secretions. Urine is rarely found deposited with other odors when used for urine marking (Asa 1993; Asa *et al*. 1985; Zhang *et al*. 2005), but feces and anal sac secretions are known to be associated in a few species. In a captive wolf study, Asa *et al.* (1985) found that the alpha male expelled anal sac secretions with 9% of scats, and the beta male and alpha female expelled these secretions with 5.7%, and 3.1% of scats, respectively. Domestic dogs, *Canis familiaris,* have also been observed to expel anal sac secretions with feces (Ashdown 1968). Although the combination of anal sac

secretion and feces has not been studied in raccoons, raccoons have been observed to smear fecal material while scent-marking with anogenital rubbing (Ough 1982). Marking with anal sac secretions and feces together may, in fact, occur frequently because anogenital rubbing is the most common form of scent-marking in raccoons and often results in a feces-like discoloration of the marked substrate (Kent 2009: Chapter 1). Furthermore, it is possible that urine as well may be deposited in anogenital rubbing, and it is with this mixture of odors that the behavior is useful for olfactory communication. Therefore, in my study, the separate presentation of feces and anal sac secretions may have not adequately represented how raccoons might encounter or use such odors for communication in the wild.

Conversely, it may also be that individual odors are not present in the feces of the raccoon. Golden hamsters (Johnston *et al*. 1993) and Djungarian hamsters, *Phodopus compelli* (Lai and Johnston 1994) have been found in habituation-discrimination tests to discriminate individual differences in the odors of feces. However, Mongolian gerbils (Halpin 1974), Eastern chipmunks (Keevin *et al*. 1981), prairie voles (Newman and Halpin 1988), red pandas, *Ailurus fulgens* (Li and Wang 2006), and river otters, *Lontra canadensis* (Rostain *et al*. 2004) have not been found to discriminate individual differences in the odors of feces. Nonetheless, feces may still be important for chemical communication more generally, as it has been found to communicate sex differences in Djungarian hamsters (Lai *et al*. 1996), meadow voles, *Microtus pennsylvanicus* (Ferkin and Johnston 1995), and river otters (Rostain *et al.* 2004). It also has been observed to be

used for marking in a fellow procyonidae the ringtail, *Bassariscus astutus,* although the function of this marking is not known (Barja and Lista 2006).

The negative results for anal sac secretion are more surprising considering that chemical and behavioral tests in other species have identified these odors as distinctive and discriminable. In beavers, *Castor canadensis,* variations in the chemical compositions of anal gland secretions carry information that allows for sex determination, individual discrimination, and discrimination of kin from non-kin (Schulte *et al*.1995; Sun and Müller-Schwarze 1998a, b). Chemical profiles of the anal gland secretions of Siberian weasels, *Mustela sibirica*, and steppe polecats, *Mustela eversmanni*, show possible coding for recognition of species, sex, age, and individuals (Zhang *et al*. 2002; Zhang *et al*. 2003). The Indian mongoose, *Herpestes auropunctatus,* and African dwarf mongoose exhibit the ability to discriminate individual odors in the anal gland secretions of conspecifics based on the relative concentrations of six short-chain carboxylic acids produced by the resident bacterial flora in the glands (Rasa 1973; Gorman 1976). Likewise it has been suggested in red foxes, *Vulpes vulpes*, that bacterial fermentation may be contributing to individually discriminable odors in their anal sac secretions (Albone and Percy 1976).

In my study, the anal sac secretion odors did not elicit a strong or consistent response from the raccoons. Interestingly, in behavioral tests with beagles, Doty and Dunbar (1974) found the same result. Though anal rubbing is a common behavior in dogs, all subjects in odor discrimination tests spent more time investigating the odors of urine of either sex than they spent investigating the odors of anal sac secretions or vaginal

secretions. Further chemical tests showed that the constituents of anal sac secretions in dogs do not vary between sexes or even vary significantly between dogs and coyotes (Preti *et al*. 1976). In a study of the chemical constituents of wolf anal sac secretions, Raymer *et al*. (1985) found a few chemical compounds that varied with gender and endocrine status, and found an implication of compounds being a product of microbial action. However, among intact males and females, castrate males, and ovariectomized females there were no differences in the levels of short-chain carboxylic acids in the anal sacs. Overall, the anal sac secretions of canids show much lower variation than the glandular secretions of other carnivores that have been studied.

Apart from Ursids, only four species of carnivores lack anal sacs, and two of these four species are in the family procyonidae, the coati, *Nasua narica* and the kinkajou, *Potos flavus* (MacDonald 1985). The anal sacs of raccoons are canid-like (Mivart 1882), but the glands in their anogenital region are much simpler than those of other carnivores, perhaps even canids. Additionally, many carnivore species also have anal pouches or auxillary anal glands which raccoons lack. Such is the case for dwarf and Indian mongooses, two of the few species that have behaviorally demonstrated individual discrimination of anal gland secretions (Rasa 1973; Gorman 1976). The mongooses have a hairy involution of circumanal integument that contains glandular pits and gathers secretions from enlarged sebaceous glands and hosts a wide variety of bacteria (MacDonald 1985). In addition to anal sacs, canids have also been described as having anal glands, a microscopic band of grape-shaped glands which open into the anal canal (Bradley and Grahame 1948). The secretion of the anal glands in the dog is mostly fatty

and may be more important for facilitating defecation than for marking (Trautmann and Fiebiger 1957). Anal glands have not been described in raccoons (MacDonald 1985). With significantly less-extensive and less complex glandular regions than mongooses, it is not unreasonable to suggest that the anal sac secretions of raccoons may also contain less-complex information than that found in the mongoose. Further, as the anal region of raccoons may even be simpler, or at least equivalent, to that of canids, the secretions of which have been shown chemically and behaviorally to not contain significant variation and to not elicit a strong response in test subjects, the anal sac secretions of raccoons may not be individually discriminable.

In conclusion, raccoons can discriminate individual differences in urine odors and these differences are likely due to genetic or diet differences between individuals. These individual odors could allow for individual recognition of litter-mates, mating partners, and coalition partners. However, further studies are needed to determine if raccoons can discriminate kinship from odor differences, thus confirming a genetic basis for individual odors. Though these experiments did not provide evidence that raccoons can discriminate between odors in feces or anal sac secretions, these may still be important odors for chemical communication. Further study is needed to determine what type of information feces and anal sac secretions carry in the raccoon. Future studies on scent-marking, communal latrine use, and spatial distribution in the wild are also needed to determine the ecological function of each odor source.

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Chapter 3: Analyzing Individual Differences in Urine Chemical Profiles

Introduction

The chemical profiles of odors have been found to contain information conveying species, gender, seasonality, age, individuality and relatedness. Sun and Müller-Schwarze (1998a) postulated that this information is coded in two general forms: qualitative and quantitative differences. Qualitative differences appear as the presence or absence of chemicals that may potentially be used for communication. For example, the flank gland compounds of the shrew, *Crocidura russula,* show qualitative differences among individuals. In the flank gland secretions, a few compounds are common to all males but each individual's composition of chemicals fluctuates largely with the seasons. As a result, this variation allows for discrimination among populations and familiar vs. unfamiliar males (Cantoni *et al*. 1996). Quantitative differences consist of varying levels of common chemicals between individuals. In gas chromatographic analyses of the scent-marks of lemurs, *Lemur catta* (Palagi and Dapporto 2006) and marmosets, *Callithrix jacchus* (Smith *et al*. 2001), high levels of similarity have been found in the chemical constituents of marks (i.e. little qualitative difference). Nonetheless, individually distinct profiles have been found by comparing relative concentrations, or quantitative differences of diverse compounds. Similarly, in the Siberian weasel, *Mustela sibirica* and steppe polecat, *M. eversmanni,* the relative abundance of several compounds differed significantly as a function of gender and age (Zhang *et al.* 2002).

These two forms of coding are not mutually exclusive. Sun and Müller-Schwarze (1998a,b) found both qualitative and quantitative differences when analyzing signals of

genetic relatedness and social group membership in the anal gland secretions of beavers, *Castor canadensis*. Similarly, wolves, *Lupus lupus*, were found to have specific urinary compounds unique to the wolf and closely related canids, as well as qualitative and quantitative differences in urinary compounds based on gender, season, and hormone levels in both males and females (Raymer *et al.*1984; Raymer *et al.* 1986). Zhang *et al*. (2005) found one male- specific compound (i.e. qualitative) in the urine of ferrets, *Mustela furo*, as well as multiple compounds significantly more abundant in males as compared to females (i.e. quantitative). Sun and Müller-Schwarze (1998b) suggest that by combining the two coding methods, the potential amount of information contained in one odor signal could be staggering.

For an animal to accurately and consistently communicate more specific information, such as individual identity, odors must contain high levels of complex information. Whether or not individual discrimination occurs is a fundamental factor affecting the patterns of sociality of a species (Bowers and Alexander 1967). What is currently known about raccoon sociality is that males and females have overlapping home ranges (Stuewer 1943; Kamler and Gipson 2003; Walker and Sunquist 1997; Gehrt and Fritzell 1997). At high and low densities, males form coalitions, defend a territory communally, and share resources, dens and possibly access to females (Gehrt and Fritzell 1998; Pitt *et al.* 2008). The benefits to raccoons of having discriminable individual odors would be the ability to recognize kin to avoid inbreeding, to recognize individual coalition partners and mating partners, to recognize non-coalition males when encountered in their home range, and to react appropriately to dominant individuals based

on remembered previous interactions. Possible sources of individual odors for raccoons include urine, feces, and anal sac secretions. Familiarization-discrimination experiments with raccoons have confirmed only the ability to discriminate odors in urine (Kent 2009: Chapter 2). Thus urine is the most reasonable odor source to examine in the search for qualitative and/or quantitative chemical differences that would allow for individual discrimination.

The chemical composition of urinary odors has been described in a wide range of animals from mice, *Mus musculus* (Schwende *et al.* 1986) to wolves (Raymer *et al*. 1984) and chemically, individually-distinct urine odors have been found in mice (Singer *et al*. 1997), rats, *Rattus norvegicus* (Singh *et al.* 1987), and ferrets, *Mustela furo* (Zhang *et al*. 2005). Mice and rats are the only species in which unequivocally genetically-distinct urine odors have been found and shown to be behaviorally discriminable (Singer *et al*. 1997; Singh *et al.* 1987).

To be individually discriminable, odors must contain a complex profile of compounds that meet the characteristics necessary for reliable cues of individuality. A cue is considered reliable when it is: 1) sufficiently distinctive that it can be easily and reliably disentangled from all other types of information, 2) highly diverse among individuals, and 3) temporally stable (Thom and Hurst 2004). The latter two characteristics can be addressed by comparing the size of the variation in the odor components within an individual and between individuals (Sun and Müller-Schwarze 1998b). This variation may be expressed as qualitative or quantitative differences and may be affected by gender, health, diet, or season, as well as genetic individuality. Many

of the volatile compounds found in urine are metabolites and can be traced back to specific organs or dietary components (Halpin 1980; Zhang *et al*. 2005). Therefore, individuals from different environments and with different diets are expected to show qualitative differences in the chemical profiles of their urine; while individuals on similar diets may only show quantitative differences in common metabolites. If individual discrimination were based on metabolic cues, animals would require periodic updating of the signals to maintain recognition. While this requirement is reasonable for densely populated animals, it is unrealistic for solitary or widely-dispersed animals (Hurst and Beynon 2004). Raccoons have been found to use communal latrines and to live in over-lapping home ranges where a group of two or three males may share a territory that overlaps with the home ranges of one to four females. Thus raccoons could frequently update metabolic signals and thus retain individual recognition of the occupants of their home range. In addition they could then use this knowledge to discriminate familiar vs. unfamiliar individuals although not non-kin from unfamiliar kin.

In this study, I analyzed the urine of nine wild-caught male raccoons to determine whether their chemical profiles have sufficient variability for individual discrimination. If there is sufficient variability, then I would expect to find greater qualitative and/or quantitative differences between individuals than within individuals. This would indicate that urine odors are possible sources of individual information in the raccoon.

Methods

Odor Sample Collection

Urine samples were collected from wild-caught male raccoons from July 2008 to April 2009 by the veterinary staff at the St. Louis Zoo. Animals were anesthetized with a ketamine injection and urine was collected through cystocentesis, inserting a needle through the body wall into the bladder. Urine was placed in plastic centrifuge tubes, sealed, and frozen at -20 °C immediately after collection.

Gas Chromatography

Each urine sample was individually thawed; 3 mL of the urine was transferred to a 20-mL vial, which was promptly sealed with a rubber septum. Then a 75-µm fused silica Solid Phase Microextraction (SPME) fiber coated with Carboxen/polydimethysiloxane (CAR/PDMS) was inserted through the septum and exposed to the air within the sealed vial. PDMS fibers were exposed to samples for up to 17 hours at room temperature (mean = 12 hours, range 8 - 17 hours.) Twelve hours at room temperature provided consistent absorption of volatile compounds by the fiber as well as time for the compounds to reach equilibrium within the vial. After the exposure period, the fiber was immediately injected into an HP5890 gas chromatogram fitted with a flame ionization detector (FID) and a fused-silica capillary column coated with 5% phenyl, 95% dimethylpolysiloxane (J&W DB-5ms, 30m, 0.25mm ID, 0.25µm df). The injector and detector were set at 250 °C and a temperature program was run with an initial time of 3 min at 50 °C then an increase to 230 °C at 5 °C per minute and a final

time of 3 min at 230 °C. The carrier gas was helium maintained at 10 psi inside the column and the machine was run in splitless mode. All samples were run twice consecutively. A total of nine samples were run, resulting in a total of 18 Gas Chromatography (GC) spectra obtained.

Data Analysis

All peaks shared by at least three individuals were selected for quantitative comparison and peak areas were integrated using Hewlett Packard ChemStation. The area of each peak was then divided by the total peak area in the spectrum giving a measure of relative concentration of each chemical or group of chemicals represented by a peak. Chemicals for which relative concentrations were < 0.01% were omitted due to unreliable quantification at such low amounts as suggested by Smith *et al*. (2001). For the two runs of the same sample, these percentages were averaged, the standard deviation between them calculated and a relative standard deviation or RSD calculated (Sun and Müller-Schwarze 1998b, Zhang *et al.* 2003). This gave nine RSD values for within-individual variation.

RSD = (SD/mean) x 100

If the two GC runs were identical, RSD values should be zero. The higher the RSD value, the greater the variation in relative concentration of that compound between the runs. Because GC runs using temperature programming naturally vary slightly, it is

reasonable to find RSD values of up to 10 when comparing runs of the same sample (Zhang *et al.* 2003).

In order to compare among individuals, all 18 GC spectra were randomly sorted into non-matching pairs, and RSD values were calculated for each pair. This gave nine RSD values for between-individual variation. High variation in the relative concentration of a compound between individuals indicates that though this compound is shared, it varies greatly quantitatively. Quantitative differences in a compound between individuals indicates it as a potential carrier of information. The within-individual RSD values were compared to the between-individual RSD values using a Wilcoxon matched pairs test in SPSS® 17.

Results

In the urine of the nine individuals, 54 peaks in the GC spectra were selected for quantitative comparison (Figure 1). Fifty percent of these peaks were shared by all individuals. Within the other 50%: 16.7% of the peaks were shared by all but one individual, 11.1% were shared by all but two, 0.07% were shared by all but three, and 14.8% were shared by five or fewer individuals. Peaks 18, 19, and 45 had the most consistent relative concentrations between individuals with RSD values of 23.99, 23.64, and 20.48 respectively.

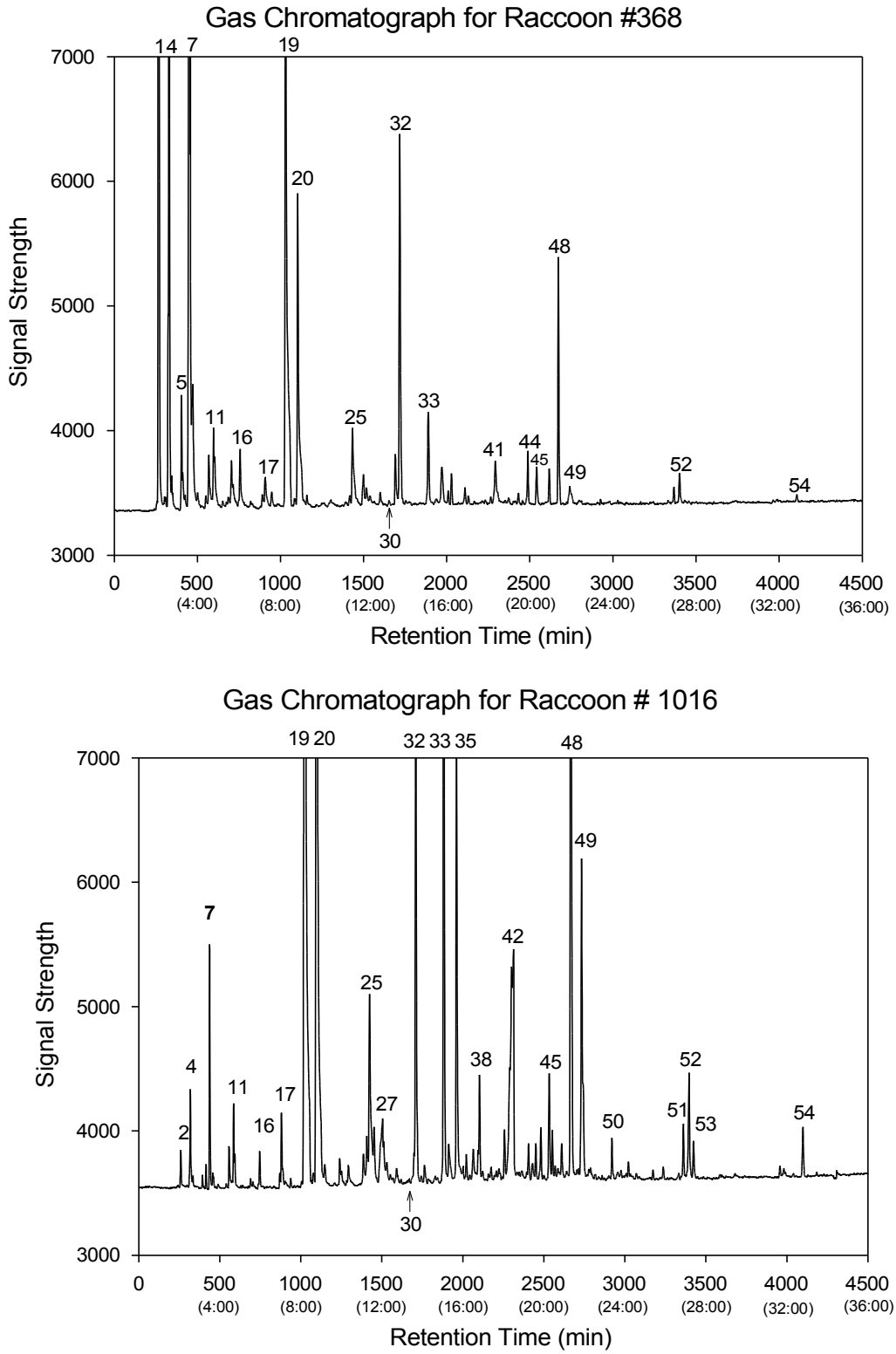

Figure 1. Gas chromatographs of volatile compounds in two individuals' urine with major peaks numbered.

Mean RSD values on the individual level (i.e. between GC runs of the same sample) ranged from 11.69 to 44.99 (mean = 25.66) with peak 33 showing the least variation and peaks 4 and 26 showing the greatest variation between runs. Mean RSD values on the group level (i.e. comparing between individuals) ranged from 20.48 to 111.44 (mean = 55.49) with peak 45 showing the least variation and peak 30 the greatest. There was significantly greater variation between individuals than between GC runs of the same individual's urine (Wilcoxon matched pairs test $p < 0.0001$, $n = 53$) (Figure 2).

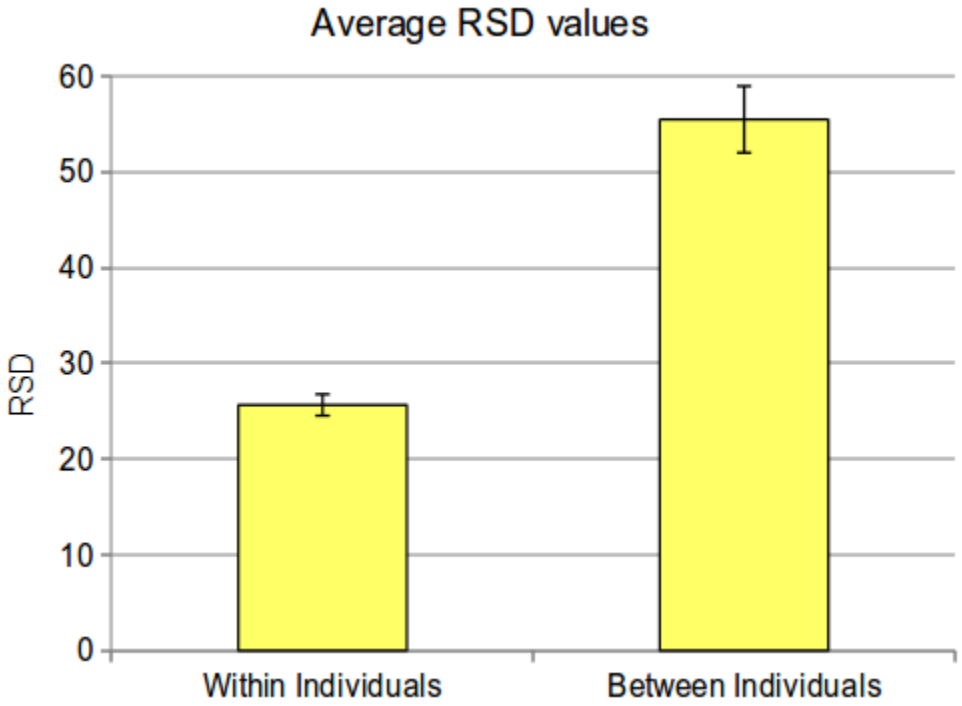

Figure 2. The mean RSD values between the GC runs of one individual's urine sample were significantly lower than the mean RSD values between individuals (Wilcoxon matched pairs test $p < 0.0001$, $n = 53$).

The peak with the lowest variation, peak 45, was shared by all individuals. However, peak 30, with the greatest variation, was only shared by six out of nine individuals. Of peaks shared by all individuals, peaks 2, 25, and 32 showed the greatest

variation with RSD values of 90.19, 79.43, and 109.13 respectively. These same peaks showed a minimal amount of variation between multiple GC runs of the same individual's urine with average RSD values of 28.14, 16.35, and 31.76 respectively.

Discussion

The RSD values between individuals in this study were similar to those found by Zhang *et al.* (2003) who documented RSD values between 33.45 and 207.89 among individuals. Their RSD values between runs of the same sample were slightly lower than those found in the present study with values of < 10 for all but three peaks. The lowest RSD value reported in this study was 11.69. The wider range of variation between GC runs reported here was most likely due to the variation in fiber exposure time between runs which affected signal strength and thus peak area. With a high level of baseline variation within GC runs of the same sample, a similarly high level of variation among individuals would not be surprising. However, since the observed variation among individuals is significantly higher than this baseline variation, it is clear that the differences I found among individuals are due to chemical differences in the samples and not merely influences of the machine or methodology.

In my study, 50% of peaks showed qualitative differences among individuals and all peaks showed a greater quantitative variation between individuals than between GC runs of a single individual. This trend of larger differences occurring between individuals as compared to within individuals is consistent with the findings of Andersen and Vulpius (1999) with lion urine, Buesching (2002) with badger subcaudal glands, and Sun

and Müller-Schwarze (1998b) with beaver anal gland secretions. These authors conclude that if quantitative variation in GC profiles is smaller within an individual than between individuals, then that odor is suitable for characterizing individuality.

Therefore, my results clearly indicate that raccoon urine is suitable for characterizing individuality because it contains variation in composition consistent with the patterns of behavioral discrimination of urine odors observed in chapter two. However, this finding does not directly indicate the presence of information in urine sufficient for individual recognition, defined as the learned discrimination between conspecifics (Halpin 1986). Urine has a large potential for conveying information on individual variation because it contains both fixed (genomic) and variable (metabolic) factors. Metabolic fluctuations resulting from health, reproductive status or diet may alter an odor signal while fixed information reflects genetically fixed characteristics such as species, gender, and individuality (Thom and Hurst 2004). In my study, all odor donors were healthy males whose diet and habitat were highly similar because they all came from a relatively small geographic area. Therefore, the qualitative and quantitative differences observed in the urine samples are most likely due to age, season and/or individuality, which could include genetic differences.

As a semi-social species, raccoons are likely to only need to recognize a moderate number of individuals in their lifetime. These individuals include their mother, litter-mates, offspring, coalition partners, and mating partners. Thus it is not necessary for every raccoon to have an invariant genomic signal in order for individual recognition to occur. Individual recognition is still possible if an incidental cue such as urine provides

enough information (quantitatively or qualitatively) to identify all individuals in a small population as well as discriminate unfamiliar individuals from familiar ones (Thom and Hurst 2004). Given the natural history of raccoons, the variation observed among individual chromatograms could easily be of sufficient complexity to allow for individual recognition of conspecifics within a raccoon's home range, even though these odor signals might shift with diet or season. The variation is also sufficient to allow for discrimination of unfamiliar individuals.

What remains to be determined is whether these odor signals also carry some genomic information that may be used for both individual recognition and kin discrimination. Genetically-derived odors, linked to the MHC region, have been found in the urine of mice (Yamazaki and Beauchamp 2007), and there exists preliminary evidence that raccoons behaviorally discriminate kin (Gehrt *et al*. 2008). Therefore, it is very likely that the urine odor of raccoons does contain genetically-derived volatile compounds. More tests with subjects and odor donors of known genotype is necessary to further confirm an odor-genes connection.

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